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ISOLATION AND CHARACTERISATION OF *XANTHOMONAS CAMPESTRES* FROM PLANT SOURCES.

Elutade O. O. and Jideani I. A.
Biological Sciences Programme, Abubakar Tafawa Balewa University Bauchi, Nigeria.

ABSTRACT

A study was conducted to isolate the ease of isolating the bacterium Xanthomonas campestris from plant sources and the cultural characteristics of the isolates. The leaves of eight different plants namely: rice (Oryza sativa), tomatoes (Lycopersicon lycopersicum), beans (Vigna unguiculata), sorghum (Sorghum bicolor), pepper (Capsicum annum), Soyabeans (Glycine max), cabbage (Brassica oleracea var. capitata), and mango (Magnifera indica), showing visible symptoms of xanthomonas bacteriosis were obtained from farm fields in Bauchi and screened on glucose nutrient agar (GNA) for Xanthomonas campestris. From the preliminary screening nine bacterial isolates B1, B3, B4, CB1, CB2, CB3, CB4, CB5, and CB6, were selected on the basis of being yellow pigmented and Gram-negative rods. These isolates were physiologically and biochemically characterised by standard determinative procedures. Only isolate B3 obtained from beans (Vigna unguiculata) was identified as Xanthomonas campestris, on the basis of its Gram-negative reaction, yellow pigmentation, mucoid growth on GNA, requirement of oxygen, production of hydrogen sulphide from sodium thiosulphate and peptone, acidification of carbohydrates, lack of urease, motility and ability to hydrolyse starch, casein and gelatin. Xanthomonas campestris B3 isolated had an optimum sodium chloride tolerance of 2% and a maximum of 8%, acid-tolerant (pH 4.5), grows rapidly at or near neutral or alkaline pH, could not grow at 4°C but a rapid growth at 37°C, and a relative growth decline at 40°C. Xanthomonas campestris B3 can form a good starter culture for the production of xanthan.

Key words: *Xanthomonas campestris*, plants, Glucose Nutrient Agar, xanthan.

INTRODUCTION

Xanthomonas Campestris, a Gram-negative bacterium originally found as a plant pathogen (Drahovska and Turner, 1995), and a very complex species (Berthier *et al.*, 1993, occurs mostly in tropical and sub-tropical areas of the world (Mooter and Swings 1990). This organism also produces an extracellular polysaccharide called xanthan, which has many applications in the food, cosmetics and oil industries (Sandford and Baird, 1983) in the developed countries.

There has not been any work in Nigeria on the cultural characteristics of *Xanthomonas campestris* or on aspects dealing with the beneficial exploitation of this organism with respect to its extracellular polysaccharide and its suitable application to the local industrial environment. The various isolations of this organism have focused on areas dealing with plant pathology (Ikotun, 1984). The exploitation of extracellular product from *X. campestris* in developing countries would require investigation into the case of isolating the organism from local sources, and the examination of cultural characteristics. The aims of this study were to screen plant sources for *X. campestris*, characterise the bacterial isolate(s) on the basis of morphological, cultural, physiological and biochemical characteristics, and obtain a starter culture of *X. campestris* for future fermentation studies towards xanthan production.

MATERIALS AND METHODS

Collection of leaf samples and preliminary screening of diseased leaves

Three or four diseased leaves were collected from each plant showing visible symptoms of the disease xanthomonas bacteriosis. The description of the diseased plant leaf samples selected including the visible symptoms shown, location and month of collection from different farm fields in Bauchi town are shown in Table 1. Collections were made at the period of matured development of the disease in the plants. The leaf specimens were processed on the day of collection. Preliminary screening investigation was carried

Table 3 Morphological, physiological and biochemical characteristics of the selected bacterial isolates compared with that of *Xanthomonas campestris*.

		Isolates[1]								
Characteristics	**XC[2]**	**B1**	**B3**	**B4**	**CB1**	**CB2**	**CB3**	**CB4**	**CB5**	**CB6**
Morphological characteristics										
Cell morphology	Db	Db	Db	Db	Db	Db	Db	Db	Db	Db
Physiological Characteristics										
Growth at 35°C	+	+	+	+	+	+	+	+	+	+
Mucoid growth on GNA	+	+	+	+	+	+	-	-	-	-
Motility	+	+	+	+	+	-	-	+	-	+
Oxygen requirement	+	+	+	+	+	+	+	+	+	+
Tolerance of NaCl (%)	2-5	8	8	8	8	8	8	8	8	<2
Biochemical Characteristics										
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	-	+	-	-	-	-	-	-	-
Gelatin hydrolysis	+	+	+	+	-	+	+	-	-	-
Urease test	-	+	-	-	-	-	-	-	+	+
Catalase test	+	+	+	+	+	+	-	+	+	-
H_2S from peptone	+	+	+	+	+	+	+	+	+	+
Acid production from:										
L (+) Arabinose	+	+	+	+	+	+	+	+	+	+
D- Glucose	+	+	+	+	+	+	+	+	+	+
D (+) Galactose	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	-	+	-	-	-	-	-	+	+

1. + = Positive reaction, - = Negative reaction, Db = Diplobacilli, < = Less than
2. XC = *Xanthomonas campestris* characterictics obtained from Dye and Lelliot (1974).

out to obtain the bacterial flora on diseased leaves. The cultural procedure used was that of Pohronezny *et al.* (1990). The characteristics observed included colony appearance, shape of isolates and pigment formation.

Isolation of target organism

The isolation technique of Pohronezny *et al.*, (1990), and Paulraj and 'O' Garo (1993), were used with modifications. The diseased leaves were surface-sterilized by dipping in methanol (99.8% v/v) for 2 seconds, followed by a rinse in CaOCl (2.5% v/v) for 10 seconds. Sections (1.0 x 2.0mm) were cut with sterile razor blade from areas of chlorosis and crushed in 0.5ml distilled water with methanol-sterilized mortal and pestle. Loopfuls of the suspension were streaked on 1% glucose-nutrient agar (GNA). Plates were incubated at 35°C for 48 hours. Colonies with yellow pigmentation were Gram stained. Gram negative rods obtained from such colonies were presumed *X. campestris* (Dye and Lelliot, 1974) and purified by repeated restreaking on GNA. Each presumptive *X. campestris* isolate was grown on nutrient agar slants and stored in a refrigerator (4°C) until required.

Isolate identification and characterisation

Selected standard biochemical tests as outlined by Dye and Leilliot (1974), and the procedures described by Ogundana (1989) were adopted on each bacterial isolate presumed to be *X. campestris*. The colony and cell morphology were carefully noted. Physiological and biochemical tests performed included, motility test, NaCl toleranace, H_2S production, catalase activity, urease activity, gelatin hydrolysis, starch hydrolysis, casein hydrolysis, and acid formation from carbohydrates. The test results were compiled and compared with the description in standard literature, that is, those for *X. campestris* (Pammel) Dowson as described by Dye and Lelliot (1974). All tests were carried out in duplicates.

Effect of NaCl, temperature, and pH on growth of *Xanthomonas campestris* B3

The growth responses of *X. campestris* B3 in liquid media, out of the nine selected bacteria isolated were measured turbidimetrically at 490nm with a colorimeter (Jenway 6050 U.K.). The growth pattern of this organism was monitored in separate test-tubes containing 25ml peptone broth media with (i) NaCl concentrations of 0.0% (control), 1.0%, 2.0%, 3.0%, 4.0%, 6.0% and 8.0% (w/v); (ii) at temperatures of 4°, 37°, and 40°C; and (iii) at pH values of 4.5, 5.5, 6.5, 7.0, 8.0, and 9.0, respectively. For each growth factor studied, curvettes were used to measure the absorbance of growth of the broth cultures at 18 hours time intervals for 54 hours.

RESULTS AND DISCUSSION

Colony appearance of isolates obtained from preliminary screening of diseased leaves

Altogether, only ten isolates (B1, B3, B4, SG1, CB1, CB2, CB3, CB4, CB5, and CB6,) showed the characteristic yellow colony colouration presumptive of *X. campestris* (Table 2) as described by Dye and Lelloit (1974). The isolation of other colony forms besides the characteristic colonies typical of *X.campestris* from the different plant leaves showing symptoms of xanthomonas bacteriosis is not surprising. *X. campestris* is not the only bacteria that can be responsible for the symptoms of necrosis, and chlorosis in plants. Several other Gram-positive bacteria such as *Corynebacterium* and Gram-negative bacterium like *Agrobacterium, Erwinia* and *Pseudomonas* may also be responsible (Billings, 1987).

When the ten bacterial isolates were Gram-stained (Table 2), all the isolates were Gram-negative and rod-shaped except isolate SG1 from sorghum that was coccoid. Gram-negative reaction and rod-shape are the characteristics of *X. campestris* as described by Dye and Lelliot (1974). Hence, isolate SG1 was no longer screened, thus reducing the number of the selected isolates to nine.

Morphological, physiological and biochemical characteristics of the nine selected isolates

The colony features of these isolates include a size range from 0.5-1.0mm, yellow pigmentation, elevated (convex) and entire margins (except isolate B6 with elevated and serrated margins). (Table 2). *Xanthomonas campestris* is known to be Gram-negative rods, with yellow pigment, round and convex colony forms with size ranging from 1.0-5.0mm (Dye and Lelliot, 1974; Mooter and Swings, 1990). The physical and chemical properties of the pigments were not studied, but it is well known that the yellow-pigmented plant pathogens of the Pseudomonadaceae family have been unified in the genus *Xanthomonas* and the yellow pigment is a polyene compound containing bromine (Schlegel, 1990).

The reaction of the nine selected isolates to physiological and biochemical characterisation (Table 3) varied with the selected isolates. However, only isolate B3 obtained from the leaves of beans *(Vigna unguiculata)* shared the same physiological and biochemical attributes as *X. campestris* (Pammel) Dowson, described by Dye and Lelliot 91974). This isolate showed Gram-negative reaction, motile rods, yellow pigmentation, mucoid on GNA, requirement of oxygen, production of H_2S from thiosulphate and peptone, lacks urease, hydrolyse starch, casein gelatin, and acidifies carbohydrates; and hence identified as *X. campestris.*

The remaining eight isolates were strongly suspected to belong to the genus *Xanthomonas* based on the fact that they all were Gram-negative rods, yellow pigmented or showed some degree of yellow on agar, aerobic, urease negative, produced H_2S from thiosulphate and peptone water, and acidified carbohydrates. These are the attributes of the genus *Xanthomonas* as reported by Mooter and Swings (1990). More physiological and biochemical tests will be required to properly identify these organisms. Isolate B3 could not be characterized to pathovar level because physiological tests are highly limiting in characterising *Xanthomonas* (Dye, 1962).

Effect of NaCl, temperature and pH on growth of *X. campestris* B3

The optimum NaCl tolerance for growth of *X. campestris* B3 in peptone broth was 2% (Figure 1). Higher and lower concentrations of NaCl, including 0.0% NaCl (control), produced a decline in growth. *X. campestris* B3 could not grow at 4°C (Figure 2), but produced rapid growth at 37°C, and a relatively declined growth at 40°C. Many strains of *X.campestris* are known to grow at 37°C (Billings, 1987), but not at 4°C or 41°C (Mass *et al.*, 1985, and Qhobella and Claflin, 1988).

X. campestris was acid-tolerant (Figure 3) and grew slowly at acid condition (pH 4.5), as compared to the rapid growth at near neutral and alkaline conditions. The optimum pH required for the production of xanthan gum, an extrapolysaccharide, by *X. campestris* has been determined to be 7.0 (Slodki and Cadmus, 1978). Hence, *X. campestris* B3 may form a good starter culture for the production of extracellular polysaccharide such as xanthan in Nigeria. The uses of microbial polysaccharides are diverse (Baird *et al.*, 1983).

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Figure 1. Effect of different concentrations of NaCl on the growth of *Xanthomonas campestris* B3.

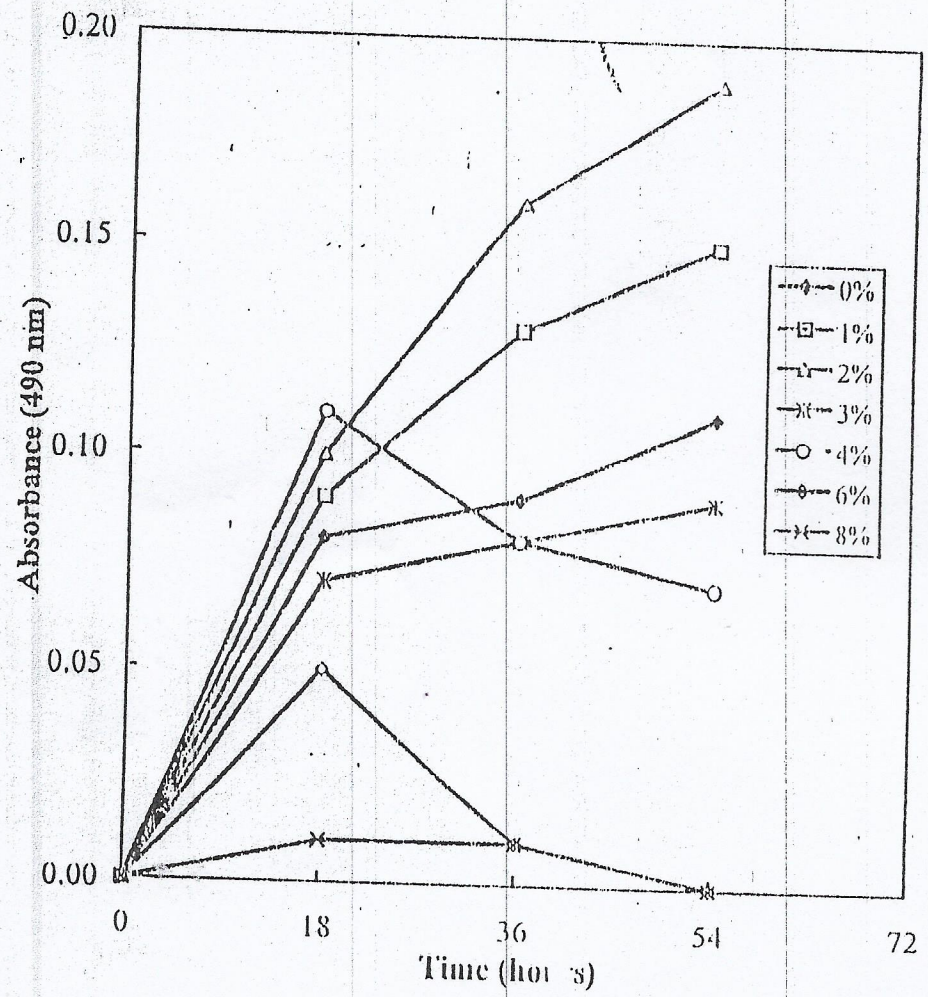

Figure 1. Effect of different concentrations of NaCl on the growth of *Xanthomonas campestris* B3.

Figure 2. Effect of different pH on the growth of *Xanthomonas campestris* B3.

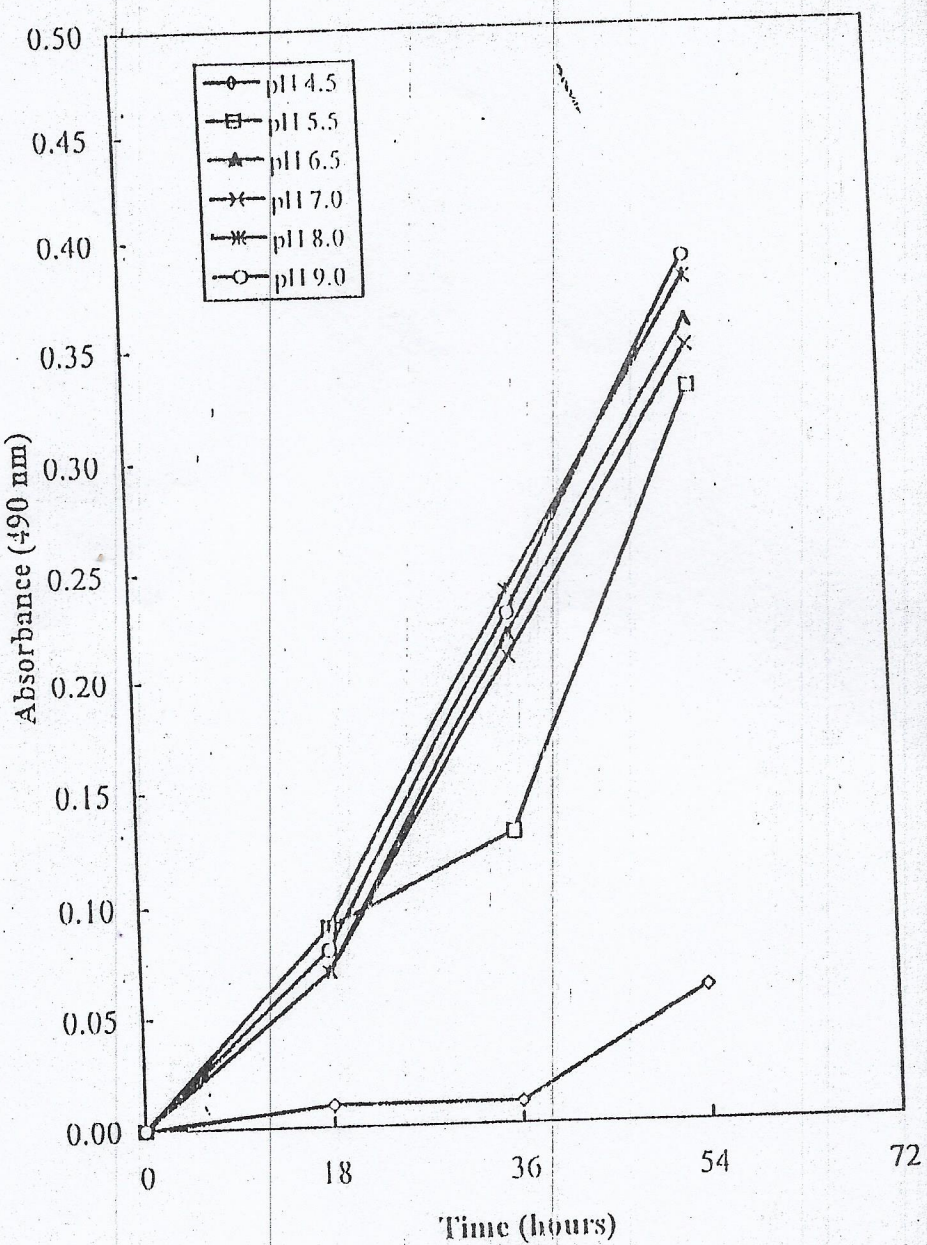

Figure 2. Effect of different pH on the growth of *Xanthomonas campestris* B3.

Figure 3. Effect of different temperatures on the growth of *Xanthomonas campestris* B3.

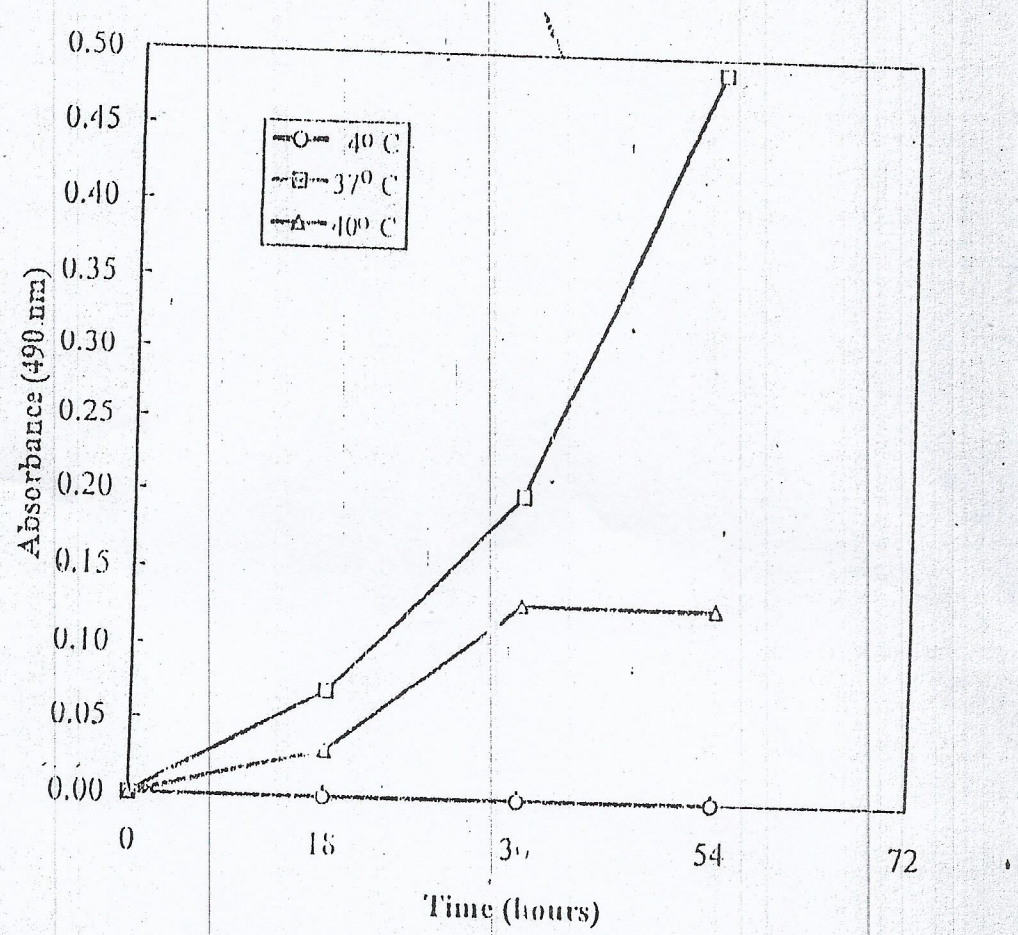

Figure 3. Effect of different temperatures on the growth of *Xanthomonas campestris* B3.

Table 1. Plant leaves collected and visible symptoms of the disease Xanthomonas bacteriosis

Plant leaf	Visible symptoms	Location in Bauchi	Period
Rice (*Oryza sativa)*	Yellow lesions along the parallel venation	Gwallameji, Yelwa and Federal Secretariat	August - October,
Sorghum *(Sorghum bicolor)*	Red-brown blotches	Gwallameji, Yelwa and Federal Secretariat	September - November,
Soya beans *(Glycine max)*	Small yellow - green spots with brown centres	Gwallameji and Yelwa	September - November,
Cabbage *(Brasica oleracea* var. *Capitata)*	Blackening of the veins with chlorosis	Wunti market	February - March
Beans *(Vigna unguiculata)*	Necrotic spot surrounded by a large	Gwallameji and Yelwa	August - November,
Tomatoes *(Lycopersicon lycopersicum)*	Small dark rough and brown lesions surrounded by a chlorotic Area	Gwallameji	September - October,
Pepper *(Capsicum annum)*	Small dark rough and brown lesions Surrounded by a chlorotic	Gwallameji and Yelwa	October - November
Mango *(Magnifera a indica)*	Black necrotic area surrounded by a chlorotic Area.	Gwallameji	August - October,

Table 2: Colony appearance of bacterial isolates obtained from preliminary screening of diseased leaves, including shape of isolates with yellow pigmentation on Glucose Nutrient Agar".

Plant leaf	Isolate	Morphology of Colony	Shape	Size(mm)	Pigmentation
Rice *(Oryza sativa)*	R4	Serrated	-	-	White
Tomatoes *(Lycopersicon lycopersicum)*	T1	Serrated	-	-	White
	T2	Serrated			
Beans *(Vigna unguiculata)*	B1	Mucoid, elevated, entire	Short rods	1.0	Yellow*
	B2	Mucoid	-	-	White
	B3	Mucoid, elevated, entire	Short rods	1.0	Yellow*
	B4	Mucoid, elevated, entire	Short rods	1.0	Yellow*
	B5	Mucoid,	-	-	White
Sorghum *(Sorghum bicolor)*	SG1	Mucoid	Cocci	-	Yellow
	SG3	Non-mucoid	-	-	White
	SG4	Mucoid	-	-	Pink
	SG5	Mucoid	-	-	Pink
Pepper *(Capsicum annum)*	P1	Serrated	-	-	White
	P2	Serrated	-	-	White
Soya beans *(Glycine max)*	SY4	Mucoid	-	-	White
	SY5	Mucoid	-	-	White
	SY6	Mucoid	-	-	White
	SY7	Mucoid	-	-	White
	SY8	Mucoid	-	-	White
Cabbage *(Brassica oleracea)*	CB1	Mucoid elevated, entire	Short rods	1.0	Light yellow*
	CB2	Mucoid, elevated, entire	Short rods	0.5-1.0	Yellow*
	CB3	Mucoid, elevated, entire	Short rods	0.5-1.0	Light Yellow*
	CB4	Mucoid, elevated, entire	Short rods	1.0	Light Yellow*
	CB5	Mucoid, elevated, entire	Short rods	0.5-1.0	Light yellow*
	CB6	Mucoid, elevated, entire	Short rods	1.0	Light yellow*
Mango *(Magnifera Indica)*	M1	Mucoid	-	-	White
	M2	Mucoid	-	-	White
	M3	Mucoid	-	-	White
	M4	Mucoid	-	-	White
	M5	Mucoid	-	-	White

a. Colony appearance with asterisks are isolates presumed *Xanthomonas campestris* and were Gram-negative rods.